Abstract: 2234031

IS A CALPROTECTIN A CALPROTECTIN: VARIATION IN CALPROTECTIN RESULTS BY METHODOLOGY

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Abstract

Background: Inflammatory bowel disease (IBD), including Crohn's disease (CD) and ulcerative colitis (UC), is a chronic disorder mediated by episodes of remittent inflammation in the gastrointestinal tract and beyond. Noninvasive testing with fecal calprotectin, a neutrophil activity byproduct, is frequently used to identify patients requiring endoscopic evaluation. While notably elevated calprotectin is highly suggestive, mild elevations are less clear, and often not linked with IBD. Multiple methodologies of testing calprotectin exist, and we hypothesized that part of the "grey area" may come from alterations to results based on methodology. We sought to assess this by comparing testing methodology on standardized patients.

Methods: We compared FDA submission data from current approved diagnostic methodologies with a novel method developed at ALPCO. We identified 76 patients diagnosed with IBD after undergoing gastrointestinal endoscopy/colonoscopy and histologic confirmation of disease. We also identified 122 patients with confirmed IBS who were diagnosed with the Rome IV criteria. We then analyzed stool samples from each of these patients using calprotectin assays and compared accuracy to previously existing endoscopic results to generate test specificity/sensitivity results.

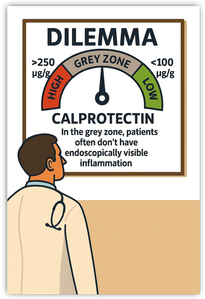
Results: Wide variation in results was observed using different methodologies, with substantial variation in sensitivity, specificity, and positive predictive value (Table 3). While the majority of assays had sensitivity > 90%, specificity ranged widely, with only four tests exceeding 90%. False positive rates were generally high, with only four assays < 10%.

Discussion: Our results demonstrate a concerning degree of variation in fecal calprotectin results by methodology. Further evaluation is needed using neutral party testing, but standardization may improve accuracy and reduce unnecessary colonoscopy.

Background

Inflammatory bowel diseases (IBD), specifically Crohn's disease (CD) and ulcerative colitis (UC), are complex immune-mediated conditions characterized by chronic remittent episodes of inflammation that manifest within and beyond the gastrointestinal tract [1, 2]. These debilitating conditions are associated with substantial morbidity and challenge healthcare systems worldwide, with rising incidence rates linked to the Westernization of lifestyle habits and dietary cues [1, 3]. While the primary site of injury is the gut, these diseases are increasingly recognized as systemic disorders with significant extraintestinal manifestations [1, 4].

In the clinical management of IBD, fecal calprotectin (fCP) has emerged as a significantly validated, non-invasive biomarker used to evaluate gut inflammation and guide therapeutic decisions [3, 5]. Calprotectin is an abundant cytosolic protein complex composed of two monomers, S100A8 and S100A9, belonging to the S100 family of leukocyte proteins [6, 7]. It constitutes approximately 45% to 60% of the total protein in the cytosol of granulocytes (neutrophils) [7, 8]. Its primary utility lies in its high sensitivity for reflecting recruited or activated phagocytes and its ability to discriminate between inflammatory conditions and functional gut diseases, such as irritable bowel syndrome (IBS) [5, 9].



Despite its utility, the clinical interpretation of mildly elevated calprotectin levels presents a significant diagnostic challenge. While levels exceeding 600 µg/g are strongly associated with active IBD, values between 100 and 250 µg/g are considered a diagnostic "grey zone" that is frequently difficult for physicians to interpret [9, 10]. These intermediate elevations are often not linked to IBD and can be triggered by viral infections, gastrointestinal bleeding, or common medications such as non-steroidal anti-inflammatory drugs (NSAIDs) and proton pump inhibitors [2, 11]. Relying on these ambiguous figures can lead to unnecessary invasive endoscopies in symptomatic patients who lack organic disease, increasing patient discomfort and overall healthcare costs (also discussed at Poster Abstract #2234024) [2, 10, 15].

In addition to the aforementioned factors that could lead to elevated Calprotectin, we hypothesize that this persistent "grey area" in diagnostic interpretation may also be driven by limitations in current calprotectin assessment methodologies and a lack of international standardization [12, 13]. Various commercial assays utilize different antibodies targeting different protein epitopes, which can lead to significant quantitative differences in reported values [13]. Furthermore, the lack of standardized guidelines for interpreting intermediate concentrations renders these results challenging for clinicians to use in daily practice [12]. This study assessed the possibility that these methodological variabilities contribute to the clinical gap between biochemical results and actual endoscopic findings [13, 14].

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Purpose

In this study, we attempt to determine whether much of discrepancies of patient samples landing in the calprotectin grey zone are caused by the different methodologies used to measure it.

Methods

Comparison of FDA approved Fecal Calprotectin Assays. We compared FDA submission data from current approved diagnostic methodologies with a novel method developed at ALPCO. For this comparison we examined:

1) The types of antibodies and calibrator materials used in each immunoassay.
2) The clinical sensitivity and specificity (with borderline samples).
3) Values achieved from various fCal assays with clinically characterized samples.

ALPCO Fecal Calprotectin Assay. We tested our assay by measuring the calprotectin from fecal samples of 76 patients diagnosed with IBD after undergoing gastrointestinal endoscopy/colonoscopy and histologic confirmation of disease. We also identified 122 patients with confirmed IBS who were diagnosed with the Rome IV criteria. We compared our accuracy to previously existing endoscopic results to generate test specificity/sensitivity results.

Results

Diversity of Antibodies and calibrator materials used in each immunoassay. As seen in Table 1, a wide mix of monoclonal and polyclonal antibodies are used by manufacturers. With the assays that use rabbit polyclonal antibodies, this could be a source of variability since these antibodies can change over time due to aging rabbits or use of different rabbits. There is also a mix use of native or recombinant calprotectin by manufacturer. Since there is no International Standard for Calprotectin, it is also challenging to know how each manufacturer calibrated their assays.

Table 1. Comparison of Antibody Types and Calibrator Material used in each 510K Cleared Fecal Calprotectin Assay.

Immunoassay	Capture Antibody Type	Detection Antibody Type	Standard/Calibrator Material	Control Material
Genova PhiCal Test	Polyclonal (Rabbit)	Polyclonal (Rabbit)	Recombinant human calprotectin	Recombinant human calprotectin
Eurospital Calprest	Polyclonal (Rabbit)	Polyclonal (Rabbit)	Recombinant human calprotectin	Recombinant human calprotectin
Eurospital Calprest NG	Polyclonal (Rabbit)	Monoclonal (Mouse)	Recombinant human calprotectin	Recombinant human calprotectin
Inova QUANTA Flash Calprotectin	Polyclonal	Monoclonal	Recombinant human calprotectin	Recombinant human calprotectin
BÜHLMANN fCAL ELISA	Monoclonal	Monoclonal	Native human calprotectin	Native human calprotectin
BÜHLMANN fCAL turbo	Polyclonal	Polyclonal	Native human calprotectin	Not specified (Traceable to recombinant)
DiaSorin LIAISON Calprotectin	Monoclonal (Mouse)	Monoclonal (Mouse)	Recombinant human calprotectin	Recombinant human calprotectin
ALPCO Calprotectin Chemiluminescence ELISA	Monoclonal (Mouse)	Monoclonal (Mouse)	Native human calprotectin	Native human calprotectin
ALPCO Calprotectin Immunoturbidimetric (IT)	Monoclonal (Mouse)	Monoclonal (Mouse)	Native human calprotectin	Native human calprotectin

Large Differences are Apparent in Calprotectin measured from the same Stools per Assay

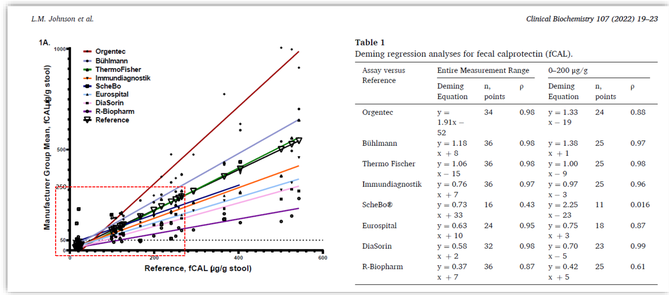

Figure 1 from Johnson et al. Clin Biochem 2022 [16] demonstrating significant differences in fecal Calprotectin values measured from the same stools. "Fig. 1. A. Deming regression plots of manufacturer group mean results for fecal calprotectin (fCAL) compared to a set of reference points (average of all manufacturer group mean results per survey). Data was gathered from INSTAND survey participants from 2015 to 2020." Red dashed box = clinically relevant sample values and the grey zone.

Challenge of Antibody use, Calibration, or Matrix? The figure above from Johnson et al 2022 demonstrates the issue at the heart of this poster. When the same stool samples are measured over various assays made by different manufacturers, a wide range of results per stool occurs. Could this be the reason for the issues related to the grey zone, could it be due to the choices in the antibodies, calibration material, and even how each company recommends extracting the stools, that such a large range then becomes possible?

Results (cont.)

Table 2. Comparison of Cut-Offs for Each 510K Cleared Fecal Calprotectin Assay.

Assay Name	Normal/Negative	Borderline/Gray-zone	Elevated/Abnormal
Genova PhiCal™ Test	< 50 µg/g	50–120 µg/g	> 120 µg/g
Eurospital Calprest®	< 50 mg/kg	50–100 mg/kg	> 100 mg/kg
Eurospital Calprest® NG	< 50 mg/kg	50–120 mg/kg	> 120 mg/kg
Inova QUANTA Flash® Calprotectin	< 50 mg/kg	50 to < 120 mg/kg	≥ 120 mg/kg
Buhlmann (ELISA & turbo)	< 80 µg/g	80–160 µg/g	> 160 µg/g
Diasorin LIAISON® Calprotectin	< 50 µg/g	50–120 µg/g	> 120 µg/g
ALPCO Chemi Calprotectin	< 50 µg/g	50–100 µg/g	> 100 µg/g
ALPCO IT (Immunoturbidimetric)	< 50 µg/g	50–100 µg/g	> 100 µg/g

Table 4. Comparison of Values of Various Manufacturers' Calprotectin Assays.

Sample #	ALPCO CLIA	ALPCO IT	Diasorin CLIA	Inova CLIA	Buhlmann IT	Clinical Diagnosis
1	194	501	148	321	756	IBD, Crohn's
2	559	413	955	575	2044	IBD, UC
3	106	403	94	221	420	IBD, Crohn's
4	415	374	545	530	1493	IBD, UC or CD
5	55	178	34	34	129	IBD, UC
6	157	131	390	412	1146	IBD, CD
7	300	99	205	190	462	IBD, UC
8	164	130	171	126	476	IBD
9	126	60	318	279	860	Other, bleeding
10	20	48	260	137	475	Other
11	58	42	76	44	244	Diverticulosis
12	33	40	117	127	338	Diverticulosis
13	8	30	6	30	26	Hemorrhoids
14	13	29	59	46	110	IBS
15	11	22	41	37	105	Other, bleeding
16	13	21	34	44	83	IBS
17	7	19	32	35	113	Other, anemia
18	3	17	6	25	14	IBS, diarrhea
19	16	13	23	51	70	Polypoid mucosa
20	6	8	14	38	63	Other
	ALPCO CLIA	ALPCO IT	Diasorin CLIA	Inova CLIA	Buhlmann IT	
Manufacturer Cut-Off	50 ug/g	50 ug/g	120 ug/g	120 ug/g	80 ug/g	
False Neg	0/8	0/8	2/8	1/8	0/8	
False Pos	2/12	1/12	2/12	3/12	8/12	

Legend: Green = sample value under the manufacturer-indicated assay cut-off; Yellow = Grey-zone/borderline; Pink = positive/above assay grey-zone.

In this cohort, the Buhlmann IT and to a limited degree the Inova CLIA yielded some samples in the clinical "grey zone" of 100-250 ug/g, that were discordant with their clinical diagnosis. The major questions are how often does this happen in the clinic and is it assay dependent?

Table 3. Comparison of Clinical Sensitivity, Specificity, and False Positive Rate with Borderline Samples Included as Positives

Calprotectin Assay Comparison Table			
Assay Name	Clinical Sensitivity	Clinical Specificity	False Positive Rate (1-Specificity)
Genova PhiCal™ Test	74.9%	94.9%	5.1%
Eurospital Calprest®	96.9%	85.0%	15.0%
Eurospital Calprest® NG	94.6%	90.2%	9.8%
Inova QUANTA Flash® Calprotectin	96.5%	78.4%	21.6%
Buhlmann fCAL® ELISA	93.3%	70.3%	29.7%
Buhlmann fCAL® turbo (IT)	91.1%	74.3%	25.7%
Diasorin LIAISON® Calprotectin	98.0%	68.8%	31.2%
ALPCO Chemiluminescence Calprotectin	92.1%	92.5%	7.5%
ALPCO IT	90.5%	93.4%	6.6%

This recalculated 510K data is critical because borderline samples are often challenging for physicians to interpret and can't be ignored. These numbers represent more clearly what should be expected in the clinic. While the majority of assays had sensitivity > 90%, specificity ranged widely, with only three tests exceeding 90%. False positive rates were generally high, with only four assays < 10%.

Calprotectin is a Calcium- and Zinc-Binding Heterotetramer of S100A8 and S100A9

Calprotectin is composed of two different EF-hand Ca^{2+}-binding proteins, S100A8 (also known as MRP-8) and S100A9 (MRP-14). Their assembly into a functional complex is highly regulated by divalent cations.

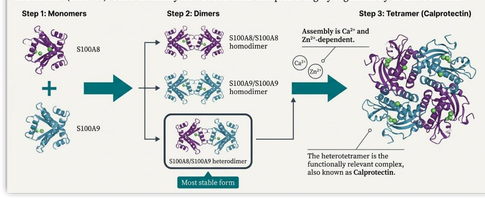

Figure 2. The many structural forms of Calprotectin. The molecule typically considered to be Calprotectin is a heterodimer made between two monomers of the proteins S100A8 and S100A9 (see box). With that said, the monomers can form homodimers themselves and in the presence of Ca^{2+} and Zn^{2+}, calprotectin heterodimers can form larger structures such as a tetramer (and possibly larger complexes). Jukic et al. [11] (see figure 3) recently showed that S100A8 and S100A9 homodimers can exist in stools of humans with active intestinal inflammation. Putting this all together, stool can contain homodimers of S100A8 and S100A9, along with heterodimeric Calprotectin and larger tetrameric structures. If an immunoassay's antibodies are able to detect the homodimers, then the level of "calprotectin" they detect will be higher than normal. Perhaps, this is contributing to the "grey zone" dilemma. Image was made with NotebookLM.

Evidence of S100A8 and S100A9 in Stool of Patients With and Without IBD

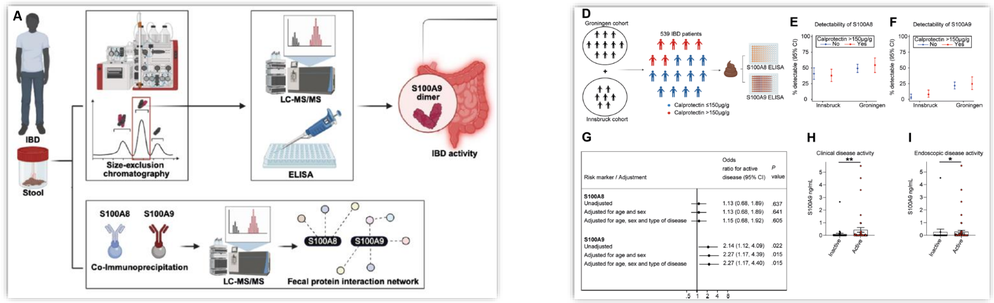

Figure 3. S100A8 and S100A9 in Human Stool. These figures come from Jukic et al.'s [11] recent article (Figure 2 in reference), in which the authors demonstrated that in humans with endoscopically confirmed IBD that via size-exclusion, mass-spec, and ELISA techniques the S100A8 and A9 homodimers can be present in stool. In a cohort of 539 patients from two sites (Innsbruck and Groningen) about 50% of calprotectin positive (>150 ug/g) subjects had S100A8 and 5-25% had A9 in their stool. What the roles are for the homodimers in IBD requires further research, but from a diagnostic perspective, this study begs the question of whether current fecal calprotectin assays can detect A8 or A9 as this may influence the results.

Table 5. *Work in Progress* - Spike of S100A8, S100A9, Calprotectin Heterodimer and Tetramer into human stool: Comparison on the ALPCO vs Diasorin Calprotectin Assays.

Calprotectin Spiked into Stool then extracted and measured on either the ALPCO Kleeya Calpro Assay or Diasorin.						
Sample ID	Stool Used for Spiking	Calpro Form Spiked	Measured Concentration [ug/g]		Normalized Recovery	
			ALPCO Kleeya	Diasorin	ALPCO Kleeya	Diasorin
PBS	Stool #1	N/A	BD	9.6		
PBS	Stool #2	N/A	NM	BD		
E. Coli Expressed S100A8	Stool #1	Monomer	BD	86.9	0%	95%
E. Coli Expressed S100A8	Stool #2	Monomer	NM	77.6	NM	88%
E. Coli Expressed S100A9	Stool #1	Monomer	BD	BD	0%	0%
E. Coli Expressed A8/A9	Stool #1	Heterodimer	28.5	93.4	48%	**100% ***
Human Native Calprotectin	Stool #1	Heterotetramer (+dimer)	96	125	**100% ***	123%

* The ALPCO assay was calibrated using native human calprotectin and the Diasorin assay was calibrated with the E. coli-made human recombinant heterodimer [13]. Both assays measure equal levels of the form of calprotectin used to calibrate them. The main difference is that the Diasorin assay also picks up the S100A8 protein which could lead to elevated Calprotectin numbers. As stated in the title, this is a work in progress. This must be repeated and tested on the other fecal calprotectin assays listed above. BD = Below Detect; NM= Not Measured.

Discussion/Future Directions

Our results demonstrate a concerning degree of variation in fecal calprotectin results by methodology. Much of this variability could be caused by antibody choice and standardization of the assay. Additionally, whether each assay detects the S100A8 or A9 monomers/homodimers requires further exploration since this could lead to higher results in patients who have those proteins, and this could contribute to the grey zone dilemma. Further evaluation is needed using neutral party testing, but standardization may improve accuracy and reduce unnecessary colonoscopy.